Cellular Stability of Serotonin *N*-Acetyltransferase Conferred by Phosphonodifluoromethylene Alanine (Pfa) Substitution for Ser-205*

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Large changes in the activity of serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AANAT) in the pineal gland control the rhythmic production of the time-keeping hormone melatonin. The activity of AANAT reflects changes in the amount and activation state of the AANAT protein, both of which increase at night. The molecular basis of this regulation is now becoming known, and recent data indicate that this involves phosphorylation-dependent binding to the 14-3-3 protein at two sites, one of which, Ser-205, is located several residues from the C terminus. In this study, we determined whether substitution of this residue with a non-hydrolyzable the phosphoserine/ phosphothreonine mimetic would promote binding to the 14-3-3 protein and enhance cellular stability. To accomplish this, a C-terminal AANAT peptide containing the phosphonodifluoromethylene alanine at Ser-205 was synthesized and fused to bacterially expressed AANAT$_{30-199}$ using expressed protein ligation. The resulting semisynthetic protein has enhanced affinity for the expressed 14-3-3 protein and exhibits greater cellular stability in microinjection experiments, as compared with the unmodified AANAT. Enhanced 14-3-3 binding was also observed using humanized ovine AANAT, which has a different C-terminal sequence (Gly-Cys) than the ovine enzyme (Asp-Arg), indicating that that characteristic is not unique to the ovine enzyme. These studies provide the first evidence that substitution of Ser-205 with the stable phosphomimetic amino acid phosphonodifluoromethylene alanine enhances binding to 14-3-3 and the cellular stability of AANAT and are consistent with the view that Ser-205 phosphorylation plays a critical role in the regulation of AANAT activity and melatonin production.

The pineal enzyme serotonin *N*-acetyltransferase (arylalkylamine N-acetyltransferase, AANAT)[1] catalyzes the acetyl transfer from acetyl coenzyme A (acetyl-CoA) to serotonin (5-hydroxytryptamine) to form *N*-acetyl-serotonin; this is the penultimate step in the biosynthesis of the time-keeping hormone melatonin (5-methoxy-*N*-acetyltryptamine) from serotonin (1). AANAT is a 24-kDa protein belonging to the GCN-5-related *N*-acetyltransferase superfamily of acetyltransferases (1, 2). Physiological changes in the cellular activity of AANAT control the daily rhythm in melatonin. AANAT activity and protein are elevated at night; in addition, light exposure during the night decreases AANAT activity, which in turn suppresses melatonin production. The molecular mechanisms underlying these dynamic changes in AANAT activity have become a topic of intensive study (3).

Evidence that cyclic AMP plays a dominant role in the regulation of AANAT activity and melatonin production has directed interest toward the role of cyclic AMP-dependent protein kinases in controlling AANAT and the possible roles of two putative protein kinase A phosphorylation sites, Thr-31 and Ser-205. Thr-31 is phosphorylated at night, which mediates binding to 14-3-3 proteins (see Fig. 1*A*) (4, 5). The family of 14-3-3 adaptor proteins has been implicated in the binding and recruiting of phosphoserine/phosphothreonine proteins to key cellular locations and in modulating their activities and functions (6). The interaction of Thr(P)-31 containing AANAT and 14-3-3 is consistent with the location of this protein kinase A site within a highly conserved consensus 14-3-3 binding motif. Physical studies have determined that Thr(P)-31 binds to the amphipathic 14-3-3 binding groove in a manner essentially identical to binding of phosphopeptides with consensus 14-3-3 binding motifs (7). The phosphorylation of Thr-31 also appears to confer cellular stability on AANAT, as indicated by substitution of Thr-31 with a non-hydrolyzable phosphoSer/Thr amino acid analog (phosphonomethylene alanine, Pma) (8). In further experiments, it was proposed that 14-3-3 recruitment mediated by Thr-31 phosphorylation was responsible for AANAT cellular stabilization (see Fig. 1*B*) (4, 5, 7, 8). Accordingly, it appears that phosphorylation of Thr-31 is an important element of the physiological regulation of AANAT.

Recently attention has focused on Ser-205. This has revealed that it is phosphorylated in parallel with Thr-31, that it promotes binding to 14-3-3, and that dual phosphorylation of both Thr-31 and Ser-205 is required for activation of AANAT. These findings are somewhat unexpected, because Ser(P)-205 is not

[1] The abbreviations used are: AANAT, arylalkylamine N-acetyltransferase; oAANAT, ovine AANAT (ovine residues 30–207 containing A200C mutation); hAANAT, humanized ovine AANAT (ovine residues 30–207 containing C200A, D206G, and R207C mutations); Pma, phosphonomethylene alanine; Pfa, phosphonodifluoromethylene alanine; MESNA, 2-mercaptoethanesulfonate-sodium salt; CHO, Chinese hamster ovarian; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography; MALDI, matrix-assisted laser desorption ionization; GST, glutathione *S*-transferase; SH, Src homology.

FIG. 1. **Design and generation of semi-synthetic AANATs to address the roles of Ser-205 phosphorylation.** *A*, the 207-amino acid AANAT protein becomes phosphorylated at Thr-31 and Ser-205 in response to the activated adrenergic receptors on the pineal gland cells (4, 5, 9). *B*, a model illustrating the role of phosphorylation and 14-3-3 recruitment in protecting AANAT protein from proteolytic destruction (4, 8). *C*, partial sequences of wild type (*WT*) AANAT and semisynthetic AANATs showing the modification (X_1) and chemical ligation site. The AANAT extreme C-terminal two amino acids (X_2X_3) that show divergence among species are also indicated. *D*, schematic diagram of expressed protein ligation (10). An N-terminal cysteine-containing synthetic peptide that contains the modification of interest reacts with the α-thioester moiety of a recombinant protein containing the AANAT-(30–199) fragment and the intein, and after intramolecular rearrangement affords a semisynthetic AANAT protein with a native amide linkage. *E*, chemical structures of phosphoserine, Pma, and Pfa. The estimated second pK_a (pK_{a2}) values (40) for phosphate or phosphonate moieties in these building blocks are indicated by their structures. *CBD*, chitin binding domain.
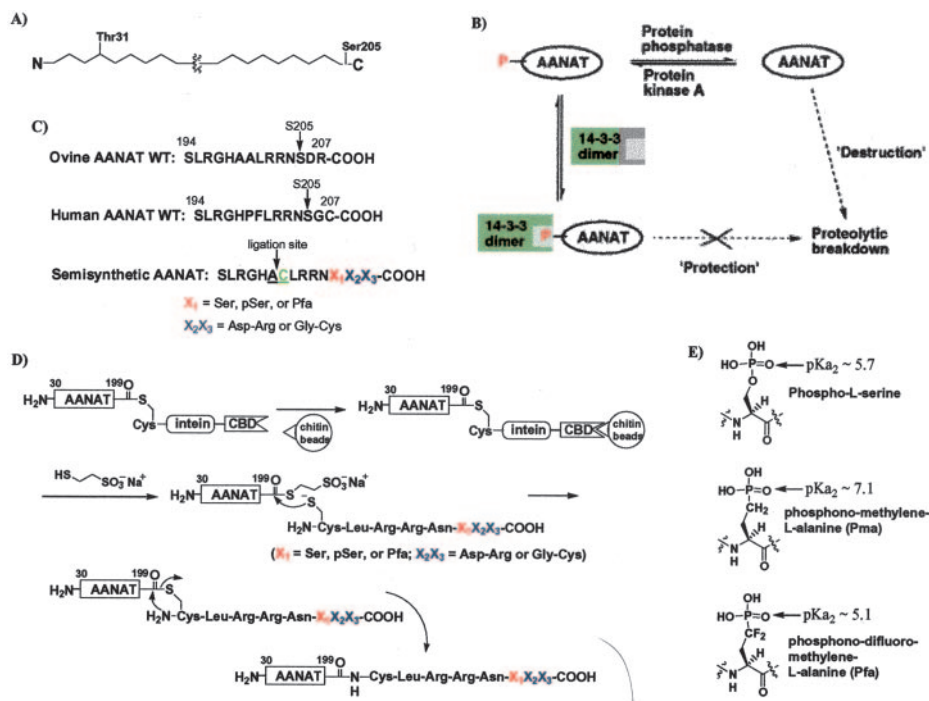
located within a consensus 14-3-3 binding motif, and the C-terminal amino acid sequence in the P+1 and P+2 positions is divergent among species (*e.g.* the P+1 and P+2 residues are Asp-Arg in ovine AANAT and Gly-Cys in human AANAT and other AANATs (see Fig. 1*C*)) (1). Although recent data indicate that Ser(P)-205 in AANAT facilitates 14-3-3 binding (9), the critical question of whether this confers cellular stability has not been addressed.

In the study presented here, we have generated a series of semisynthetic ovine AANAT (oAANAT, residues 30–207) proteins incorporating serine (oAANAT-Ser-205), phosphoserine (oAANAT-Ser(P)-205), or the non-hydrolyzable phosphoserine/phosphothreonine mimetic phosphonodifluoromethylene alanine (Pfa) (oAANAT-Pfa-205) (Fig. 1*E*) at position 205 (Fig. 1, *C* and *D*) (10, 11). In addition, the potential impact in differences in the C terminus was investigated using two semisynthetic humanized AANAT proteins (hAANAT-Ser-205 and hAANAT-Ser(P)-205). Our results support the view that Ser-205 phosphorylation of AANAT enhances its affinity for 14-3-3 protein binding and enhances the cellular stability of AANAT.

EXPERIMENTAL PROCEDURES

Preparation of the AANAT Construct—The in-frame subcloning of the ovine AANAT-(30–197) cDNA into the pTYB2 vector (New England Biolabs) using NdeI and SmaI restriction sites has been described previously (12). By using QuikChange site-directed mutagenesis (Stratagene), the two extra residues (Pro-Gly) from the SmaI restriction site in this construct were replaced with the His-Ala that are present as residues 198 and 199 in the wild type ovine AANAT sequence, affording the new plasmid pTYB2-AANAT-(30–199) used in the present study. This construct was verified by complete DNA sequencing. Note that this ovine construct was used for both the generation of the ovine and humanized C-terminal sequences in expressed protein ligation.

AANAT Acetyltransferase Activity Assay—These assays were carried out using the spectrophotometric assay described previously using saturating (>5 times K_m) acetyl-CoA and tryptamine concentrations (2).

Preparation of Fmoc-protected Pfa—Following the method of Berkowitz *et al.* (13), the intermediate, (*S*)-*N*-(*tert*-butyloxycarbonyl)-3-[1′,1′-difluoro(diethylphosphono)methyl]alanine was prepared from Garner ester as reported (14). Pfa was obtained by treating this intermediate with 1 M trimethylsilyl triflate and 2 M dimethylsulfide in trifluoroacetic acid (15). Fmoc-Pfa was obtained by standard Fmoc installation using Fmoc-Osu and the final compound purified by reversed-phase high pressure liquid chromatography (HPLC) with a C-18

column. The identity and purity of this compound was confirmed by electrospray mass spectrometry and ^{1}H NMR. The combined yield for these last two steps was 43%.

Peptide Synthesis—All standard Fmoc-protected amino acids and resins were obtained from Novabiochem, and Fmoc-Pfa was obtained as described above. All peptides were synthesized by the standard Fmoc strategy on a Rainin PS-3 peptide synthesizer. For Fmoc-Pfa, which was used in substoichiometric amounts, acetic anhydride capping was done after coupling. All synthetic peptides were purified by reversed-phase HPLC on a preparative or a semipreparative C-18 column. The purity (>95%) of the peptides was verified by analytical reversed-phase HPLC, and their molecular weights were confirmed by both matrix-assisted laser desorption ionization (MALDI) and electrospray mass spectrometric analyses.

Expressed Protein Ligation—Expressed protein ligation reactions were done as described previously (10–12, 16). Briefly, the plasmid pTYB2-AANAT-(30–199) was transformed into *Escherichia coli* BL21(DE3) cells that were grown at 37 °C until $A_{600} \sim 0.6$. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.2 mM. Cells were harvested after 20 h at 16 °C and were lysed by French press, and the cell lysates were loaded onto chitin beads, and the beads were washed as described previously (10–12, 16). The immobilized AANAT fusion protein was incubated with the ligation buffer (25 mM Hepes, pH 8.0, 250 mM NaCl, 1 mM EDTA, 200 mM MESNA, 1 mM peptide) for 3.5 days at room temperature. The ligation products were eluted off the chitin beads, and the desired fractions were pooled, extensively dialyzed to remove MESNA and unreacted peptide, and concentrated by ultrafiltration to ~1 mg/ml (determined by Bradford assay). All the semisynthetic proteins were highly pure (>90%) as revealed by 15% SDS-PAGE stained with Coomassie Blue (see Fig. 2*A*), and their correct molecular masses (±60 Da) were confirmed by MALDI mass spectrometric analyses (see Fig. 2*B*). The semisynthetic AANAT proteins were flash frozen in liquid nitrogen and stored at −80 °C in aliquots.

GST-14-3-3 Pull-down—Pull-down assays were performed as described previously (8, 16). In brief, an aliquot of 15 μl of GST-14-3-3 (~2 mg/ml) immobilized on the glutathione-agarose resin was incubated with each individual semisynthetic AANATs (7.4 μl, 1 mg/ml) and 30 μl of binding buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol) at 16 °C for 4 min. Ionic strength was adjusted by changing NaCl concentration (50 *versus* 100 mM). The incubation mixtures were pelleted, the beads were washed three times with the wash buffer at 4 °C, the resulting beads were boiled in 1× SDS-PAGE loading buffer, and all the supernatants were loaded and analyzed by 15% SDS-PAGE. Each experiment was repeated at least three times with good reproducibility.

Microinjection Experiments—CHO-K1 cells (ATCC) were cultured in ATCC F-12K medium supplemented with 10% (v/v) fetal bovine serum

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and 100 units of penicillin-streptomycin and incubated at 37 °C in the presence of 5% CO_2 (8, 16). Cells were seeded onto glass coverslips and used for microinjection within 24 h (60–70% of confluence). For individual microinjection (room temperature), semisynthetic AANATs (1.0 mg/ml in 45 mM Hepes, pH 7.0, 455 mM NaCl, 0.9 mM EDTA, 9 mM dithiothreitol, 9% (v/v) glycerol) were diluted by 2-fold into the following buffer, 50 mM Tris, pH 7.5, 25 mM NaCl. Following microinjection, cells were incubated (37 °C, in 5% CO_2, 95% air, for 60 min) and prepared for immunocytochemistry. At least 100 cells were injected with each semisynthetic AANAT in each of the three independent experiments.

Immunocytochemistry Experiments—Cells were fixed and stained with the primary rabbit antibody (AS 2819) against ovine AANAT-(1–205), followed by the incubation with a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Accurate Chemical) (8, 16). By using the IPLab software, the fluorescence intensities of all positive cells (visualized above background) were quantified, and the sum was multiplied by the number of positive cells divided by the number of injected cells, giving a relative fluorescence intensity value as the indication of the stability of the microinjected proteins inside the cells. Data are presented as the means ± S.E. The results shown are representative of three separate experiments.

RESULTS

Generation of Semisynthetic AANAT Proteins—Ovine AANAT-(residues 30–199) was subcloned into the pTYB2 vector in-frame with the intein-chitin binding domains. The N-terminally deleted construct, which is known to be well expressed and catalytically active (2), was selected to provide the simplest system for exploring the role of C-terminal post-translational modification. This eliminated the potential contribution from the 14-3-3/protein kinase A site, which centered on Thr(P)-31. Expressed protein ligation performed in the presence of MESNA as described previously (11, 12) led to a robust protein production (>2 mg/liter of *E. coli* cell culture), which appeared to be greater than 90% pure eluting from the chitin beads (Fig. 2*A*), adequate for further characterization. The resulting AANAT semisynthetic proteins had acetyltransferase activity that were within 2-fold of each other (data not shown).

14-3-3 Binding—The role of Ser-205 phosphorylation in mediating AANAT interactions with 14-3-3 was studied by *in vitro* GST pull-down assays. GST-14-3-3ζ was immobilized on glutathione-agarose resin and incubated with individual semisynthetic AANAT proteins for 4 min at 16 °C prior to several brief buffer washes at 4 °C. Under these conditions, only a modest fraction of each of the semisynthetic AANAT proteins is retained on the resin compared with the input (Fig. 3*A*). Nevertheless, it is clear that oAANAT-Ser(P)-205 and oAANAT-Pfa-205 proteins exhibited stronger association to 14-3-3 protein than semisynthetic oAANAT-Ser-205 (Fig. 3*A*). Likewise, hAANAT-Ser(P)-205 showed somewhat greater binding to 14-3-3 than hAANAT-Ser-205 (Fig. 3*B*). This difference was seen in the presence of 50 and 100 mM NaCl, but greater AANAT/14-3-3 association at 50 mM NaCl could reflect enhanced electrostatic interactions. Under the same experimental conditions including 100 mM NaCl, both oAANAT-Ser(P)-205 and oAANAT-Pfa-205 displayed stronger association to 14-3-3 as compared with hAANAT-Ser(P)-205 (Fig. 3*C*). Although these differences were not dramatic, they were highly reproducible over three separate experiments and are consistent with the results of related competition experiments reported previously (9). These findings thus indicate that C-terminal phosphorylation can facilitate 14-3-3 interaction, even though neither the ovine nor human AANAT C-terminal phosphorylation site fits the canonical 14-3-3 binding motif, *i.e.* they lack a Pro residue at the P+2 position (6, 17–20).

The relative 14-3-3 binding of semisynthetic protein ovine AANAT-(1–197)-Thr(P)-31 (8) and ovine AANAT-(30–207)-Ser(P)-205 (Fig. 3*D*) were examined; although the former appeared to exhibit stronger binding, the basis of this is not clear, it could reflect contacts specifically linked to the phosphoryla-
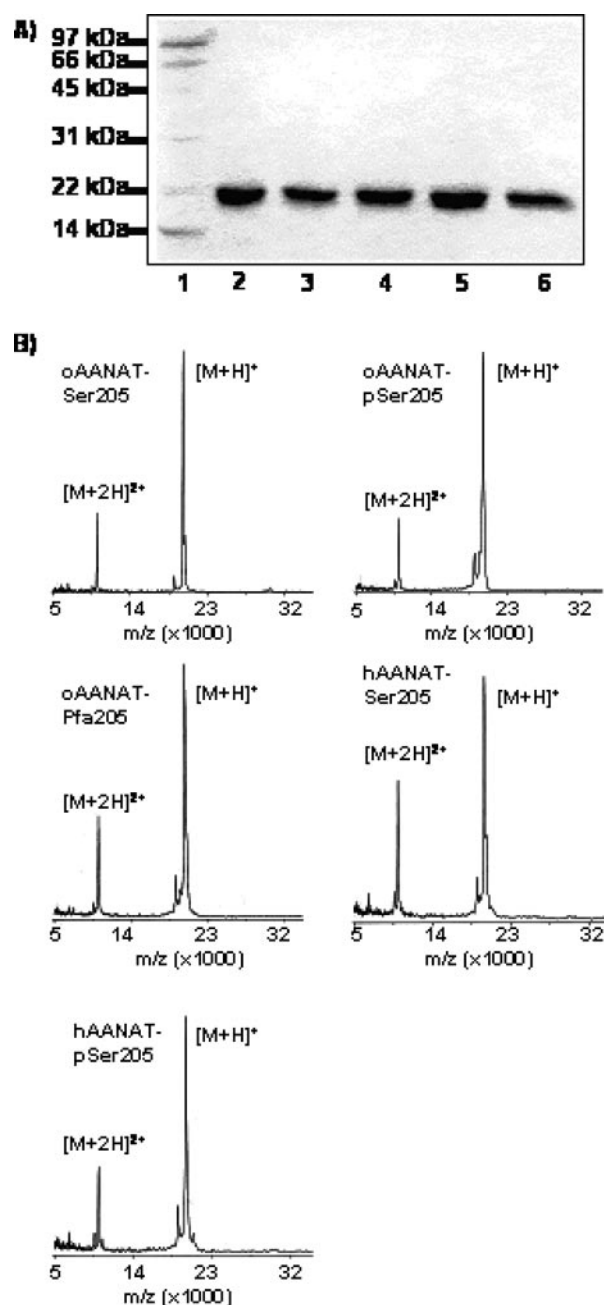
FIG. 2. **SDS-PAGE and mass spectrometry analyses of semisynthetic AANATs.** *A*, 15% SDS-PAGE stained with Coomassie Blue of purified semisynthetic AANATs. *Lane 1*, molecular mass markers; *lane 2*, oAANAT-Ser-205; *lane 3*, oAANAT-Ser(P)-205; *lane 4*, oAANAT-Pfa205; *lane 5*, hAANAT-Ser-205; *lane 6*, hAANAT-Ser(P)-205. *B*, MALDI time-of-flight-mass spectrometry profiles of semisynthetic AANAT proteins. The observed masses are within ±60 Da from the calculated masses.

tion site or to contacts involving ovine AANAT-(1–30). These and related studies indicate single phosphorylation of AANAT at either Thr-31 or Ser-205 promotes binding to 14-3-3; moreover, the binding studies indicate that Pfa is an effective mimic of Ser(P) in the context of ovine AANAT.

Effects of Ser-205 Phosphorylation on AANAT Protein Cellular Stability—The above demonstration that AANAT Ser-205 phosphorylation strengthens binding to 14-3-3 pointed to the possibility that Ser-205 phosphorylation enhances AANAT protein cellular stability, as has been established with Thr-31 modification. The apparently similar 14-3-3 binding of oAANAT-Ser(P)-205 and oAANAT-Pfa-205 indi-
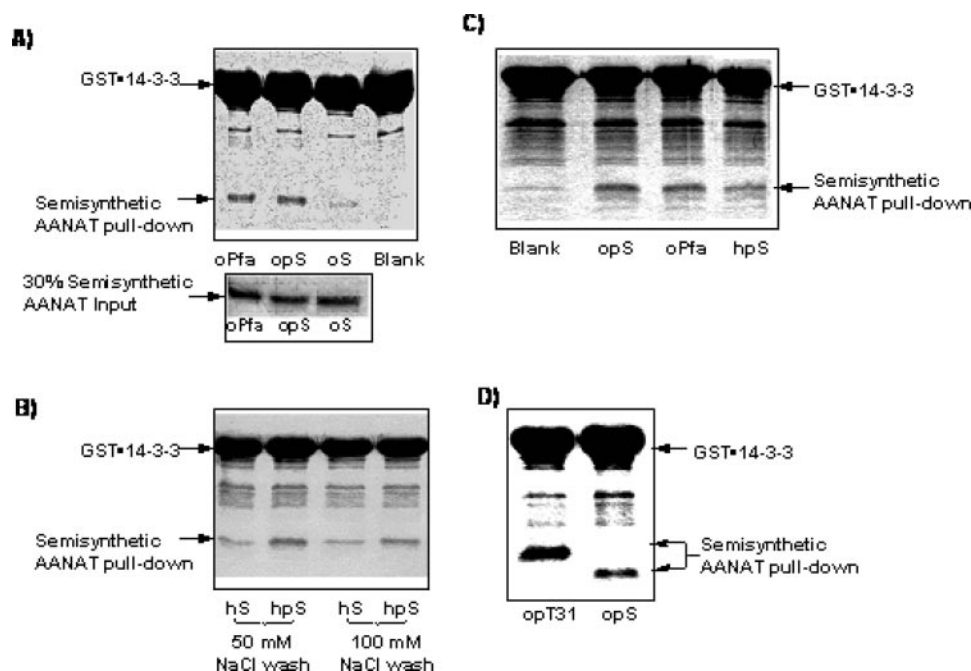
FIG. 3. **Evaluation of semisynthetic AANATs *in vitro*.** *A–D*, 14-3-3 interactions with semisynthetic AANATs as assessed by an *in vitro* GST pull-down assay and analyzed by 15% SDS-PAGE stained with Coomassie Blue. *oPfa*, oAANAT-Pfa-205; *opS*, oAANAT-Ser(P)-205; *oS*, oAANAT-Ser-205; *hpS*, hAANAT-Ser(P)-205; *hS*, hAANAT-Ser-205, *opT31*, oAANAT-Thr-31 (8); *Blank*, immobilized GST·14-3-3 only.
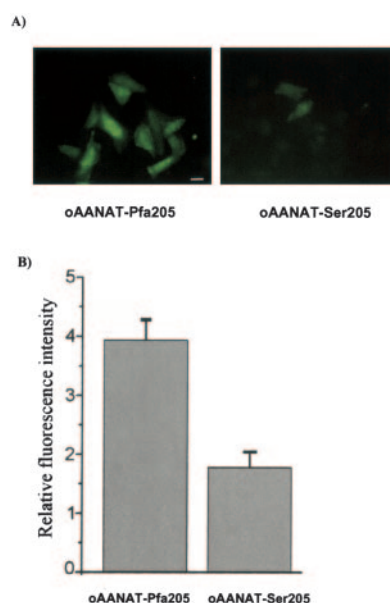

FIG. 4. **Evaluation of semisynthetic AANATs *in vivo*.** *A*, a representative presentation of fluorescence intensities in CHO cells from microinjected oAANAT-Pfa-205 and oAANAT-Ser-205 1 h after protein injection. *Bar*, 20 μm. *B*, relative stability of oAANAT-Ser-205 and oAANAT-Pfa-205 inside CHO cells as evaluated by the relative fluorescence intensities after a 1-h incubation at 37 °C following cellular microinjections. Identical concentrations (based on SDS-PAGE and Bradford assay) for oAANAT-Ser-205 and oAANAT-Pfa-205 were used in these experiments.
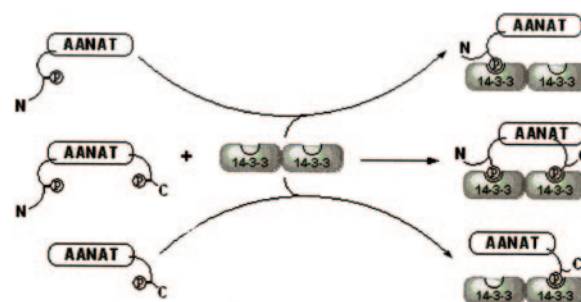

FIG. 5. **A proposed model for the interaction between phosphorylated AANAT and the 14-3-3 protein.** The C- or N-terminal monophosphorylated AANAT is expected to recruit 14-3-3 via a monovalent binding interaction mode, however both leading to enhanced AANAT cellular stability. N- and C-terminal doubly phosphorylated AANAT protein may enhance 14-3-3 recruitment through bivalent interaction, leading to further increased AANAT cellular stability.

cated that any differences in protein stability that might occur in the context of the cell would primarily reflect differences in the phosphorylation state and would not reflect dephosphorylation because of the stable nature of oAANAT-Pfa-205. Therefore, oAANAT-Ser-205 and oAANAT-Pfa-205 were each microinjected into CHO cells, which have endogenous 14-3-3 protein and intact cellular protein degradation pathways (21, 22) but do not express AANAT. The initial concentration of an injected protein inside the CHO cells was

estimated to be ~180 nM (23) that is close to the night time endogenous ovine AANAT level.[2] The relative stabilities of oAANAT-Ser-205 and oAANAT-Pfa-205 in CHO cells were compared at the 1-h time point after microinjection, as in our previous studies on AANAT Thr-31 phosphorylation (8). The intracellular concentration of injected oAANAT-Ser-205 or oAANAT-Pfa-205 was monitored by immunofluorescence using a polyclonal anti-ovine AANAT antibody raised against full-length bacterially expressed ovine AANAT. The fluorescence intensity was integrated throughout all positive cells. oAANAT-Pfa-205 generated a ~2-fold greater fluorescence intensity than oAANAT-Ser-205 (Fig. 4). Assuming that differences in fluorescence intensity reflect different intracellular degradation of oAANAT-Ser-205 and oAANAT-Pfa-205, these data indicate that the Pfa-205 modification confers intracellular stability on AANAT, possibly via 14-3-3 recruitment. Although Western blot analysis of oAANAT-Ser-205 stability would have also been desirable, the microinjection

approach made necessary by the *in vitro* semisynthesis of proteins precludes the generation of sufficient material for these studies.

DISCUSSION

AANAT protein is thought to become phosphorylated at night at two putative protein kinase A sites, Thr-31 and Ser-205, in response to the elevation of cyclic AMP produced by β- and α_1-adrenergic receptor activation of pinealocytes (Fig. 1*A*); a contribution from calcium phospholipid-dependent protein kinase is also possible (4, 5, 9, 24). Such post-translational modifications may serve as important functional switches to regulate the AANAT protein, which in turn controls melatonin production. Indeed, prior studies indicated that AANAT phosphorylation induces the recruitment of the 14-3-3 protein and acetyltransferase catalytic efficiency (4, 5, 9).

Analysis of the functional roles of AANAT post-translational phosphorylation has focused previously on the N-terminal Thr-31 residue. This is primarily related to the fact that *in vivo* AANAT Ser-205 phosphorylation has only very recently been demonstrated (9), whereas Thr-31 modification was discovered several years ago. Here we have explored the effects of AANAT Ser-205 phosphorylation on the interaction with 14-3-3 and cellular stabilization. By employing site-specific incorporation of the non-hydrolyzable Ser(P) analog, Pfa, it was demonstrated that Ser-205 modification is likely to enhance AANAT cellular stability. A simple model that accounts for these findings is that AANAT-Pfa-205 can recruit 14-3-3, which somehow protects AANAT from proteolytic breakdown. A similar proposal was made for Thr-31 phosphorylation. Taken together, these studies argue that anchoring AANAT to 14-3-3, regardless of the AANAT binding surface involved, is sufficient to slow the cellular destruction of AANAT. The significance of these findings is that they support the proposal that 14-3-3 prevents AANAT destruction by shielding it from metabolic processes that lead to proteasomal proteolysis, either by partitioning or hindering access of macromolecules to a surface of AANAT that is necessary for degradation (5). Further studies will be needed to more definitively address the molecular mechanisms leading to phospho-AANAT cellular stabilization.

Another question still unanswered is the relative contribution of multiple phosphoryl modifications to cellular stability. Other studies indicate that dual phosphorylation is required for 14-3-3-dependent activation of AANAT (9), presumably because this stabilizes a floppy loop component of the AANAT binding pocket and confers optimal arylalkylamine binding. Accordingly, both N- and C-terminal phosphorylation sites, as illustrated in Fig. 5, may engage the dimeric 14-3-3 binding surfaces and play critical roles for both activation and stabilization of cellular AANAT. It will be of interest to investigate the cellular stability of bisphosphono-AANAT, which should be accessible via sequential peptide ligation reactions (25, 26).

As discussed, AANAT C-terminal sequences show divergence among species, with the ovine enzyme terminating in Asp-Arg and other mammalian (including human) enzymes in Gly-Cys (Fig. 1*C*) (1). These amino acids occupy the P+1 and P+2 positions relative to Ser-205, and their composition may influence the consequences of Ser-205 phosphorylation. Indeed, under the same experimental conditions (*e.g.* 100 mM NaCl wash with added 0.1% Triton X-100), both oAANAT-Ser(P)-205 and oAANAT-Pfa-205 displayed a tighter binding to 14-3-3 as compared with hAANAT-Ser(P)-205 (Fig. 3*C*). It is possible that the presence of an Asp residue at the P+1 position in the ovine sequence may enhance 14-3-3 interaction by virtue of its negatively charged side-chain, which is in the vicinity of a highly positively charged Ser(P)/Thr(P) binding pocket in the 14-3-3 protein (7, 19, 20). Future structure-function analysis may

shed light on this point. However, hAANAT-Ser(P)-205 was still shown to exhibit a greater binding to 14-3-3 than hAANAT-Ser-205 (Fig. 3*B*). These results support the notion that enhanced 14-3-3 recruitment following Ser-205 phosphorylation is a general phenomenon for AANAT proteins from various mammalian species including humans.

The utility of protein semisynthesis and expressed protein ligation in particular in addressing the functional and structural roles of reversible protein post-translational modifications including phosphorylation is becoming increasingly established (8, 10, 16, 27–35). Expressed protein ligation is most conveniently applied when a modification site is located in the C terminus of a protein because a C-terminal thioester generated using inteins can be ligated with a readily prepared N-terminal cysteine-containing peptide (10). Because the post-translational modification is installed by chemical synthesis, the precise location and stoichiometry can be easily controlled. Studies analyzing phosphorylation and kinase/phosphatase action in transforming growth factor β-substrate interactions (30–32), Csk-Src regulation (10, 29, 33), SHP-1 (34), and SHP-2 (16, 35) exemplify the value of expressed protein ligation in signaling.

Because the post-translational modifications in protein semisynthesis are introduced chemically, the experimentalist is not limited to incorporating naturally occurring chemical linkages, which are susceptible to enzymatic cleavage. Replacement of the bridging oxygen in phosphate modifications with a carbon atom appears to be generally well tolerated in phosphate molecular recognition (36, 37). The recent work on SHP-1 and SHP-2 illustrates the strength of using non-hydrolyzable phosphotyrosine analogs to examine the structure and function of a signaling protein (16, 34, 35). Historically, glutamate and aspartate have been regarded as mimetics of phosphoserine and phosphothreonine for addressing the roles of the reversible serine/threonine phosphorylation. However, success with these replacements has varied (6, 38, 39). Our previous experience in addressing the regulatory roles of AANAT Thr-31 phosphorylation also demonstrated the limited value of Glu as a phosphorylated residue mimic (8). In this regard, the non-hydrolyzable phosphoserine/phosphothreonine mimetics Pma, and especially Pfa, constitute attractive alternatives. Because of the electron-withdrawing effects of fluorine atoms, Pfa more closely mimics the pK_a of phosphoserine compared with Pma (Fig. 1*E*) (35, 40). Moreover, the fluorine atoms in Pfa have the potential to be hydrogen bond acceptors, which could mimic the interactions with the bridging oxygen of the phosphate. Superior performance of fluoromethylene phosphonate mimics was observed in the context of phosphotyrosine-SH2 interaction with SHP-1 and SHP-2 (34, 35).

In summary, by employing expressed protein ligation, the non-hydrolyzable phosphoserine/phosphothreonine mimetic Pfa has been for the first time successfully incorporated into a protein, at position 205 of AANAT, the protein regulating rhythmic melatonin production. Its evaluation side by side with phosphate-modified and unmodified semisynthetic AANAT proteins suggested that AANAT Ser-205 phosphorylation enhances AANAT cellular stability possibly mediated by phosphorylation-dependent 14-3-3 recruitment.

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